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Characterization of gene expression profiles in developing kernels of maize (*Zea mays*) inbred Tex6

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Abstract

Maize inbred Tex6 is resistant to several pests. The objectives of this study were to characterize gene expression profiles in Tex6 kernels and identify unique genes that expressed up- or down-ward in developing kernels in the later stages. Because of the resistance of Tex6 kernels, we reasoned that it would be an interesting candidate for microarray study. By using maize microarray, we analysed gene expression profiles in developing kernels from 25 DAP (days after pollination) to 45 DAP. A total of 8497 positive array spots were detected with unique IDs, in which 4247 genes were detected in all samples. The trends of total expressed genes were decreasing when kernels were maturing. Expression patterns of some genes in several metabolic pathways, including starch, lipid and storage proteins, were analysed. In comparison with 25 DAP, expressions of 211 gene features were significantly different at 45 DAP ($P < 0.05$), which will be used to produce a macroarray for germplasm assessment and evaluation. The real-time qRT-PCR was used to validate microarray study.

Key words: *Zea mays* — *Aspergillus flavus* — developing kernels — aflatoxin — resistance genes — gene expression

In maize, seeds as a model system for developmental and genetic studies have been studied extensively for morphological analysis (Sheridan and Clark 1994, Bommert and Werr 2001, Consonni et al. 2005). Kernel maturation leads to a developmental endpoint in the endosperm, whereas the embryo retains the regenerative capacity after germination. Maize kernel development is divided into six stages (Ritchie and Hanway 1986): silking (R1), blister (R2), milk (R3), dough (R4), dent (R5) and physiological maturity (R6). The entire process starts with morphogenesis at early stages with cell division and enlargement, followed by a maturation phase at late stage. The genes related to starch and storage protein syntheses in maize have been extensively studied (Woo et al. 2001, Song and Messing 2003, Schultz and Juvik 2004, Gibbon and Larkins 2005).

In maize kernels, cDNA microarray has been used to investigate gene expression changes during maize kernel development (Cho et al. 2002, Fernandes et al. 2002, Lee et al. 2002, Zinselmeier et al. 2002, Grimanelli et al. 2005, Verza et al. 2005). Maize oligonuleotide arrays have been made available through the Maize Oligonuleotide Array Project (MOAP) (http://www.maizearray.org) with 58K features on a set of two slides. Cho et al. (2002) conducted gene-expression profiles among 13 samples from seven maize organs using cDNA microarray containing 5376 unique genes, and reported that only 7% of the genes were highly organ specific and the majority of genes were expressed in diverse organs with little difference in transcript levels. Unfortunately, individual gene expression profiles from cDNA array are obscured by cross-hybridization among family members (Xu et al. 2001, Fernandes et al. 2002), resulting in identification of false positive genes. In comparison though with cDNA array, oligo array can achieve hybridization patterns of transcript levels relatively accurate.

By using maize oligo-array, our goal was to study gene expression profiles in the maize kernels of Tex6 and to identify a set of unique genes that could be used as a tool in maize germplasm assessment for breeding for resistance to preharvest aflatoxin contamination (Guo et al. 2005). The maize line Tex6 was reported to have resistance to aflatoxin contamination, and resistance to several ear-feeding insects (Hamblin and White 2000, Dowd and White 2002, Moore et al. 2004, Guo et al. 2007). Several studies have focused on the early-to-middle developmental stages before 25 days after pollination (DAP) (Cho et al. 2002, Fernandes et al. 2002, Lai et al. 2004, Grimanelli et al. 2005, Verza et al. 2005), but there is a lack of information about the later stages of maize kernel development. After 25 DAP, morphological changes of kernel have finished, and the main changes appear to be metabolisms related to storage components. Although zeins as main storage proteins have been extensively studied (Woo et al. 2001), the potential relationship of gene expression of these proteins with disease resistance was not studied.

Because of the tolerance traits of Tex6, especially fungal resistance in developing maize kernels at later stages (Payne 1998), we selected this inbred for this study to characterize gene expression profiles in Tex6 developing kernels and to identify unique genes which expressed up- or down-ward in developing kernels in the later stages and could be used in germplasm assessment. In this report, transcriptional profiles of Tex6 kernels at 25, 30, 35, 40 and 45 DAP were compared using maize oligonucleotide arrays based on relative expression quantitation, in which glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was included in the original array production as an internal control. Total of 211 gene features at 45 DAP were selected from 823 defence-related genes and will be used to produce macroarray as an assessment tool of maize germplasm. Real-time qRT-PCR was also used for validation of microarray study.

Materials and Methods

Plant material and cDNA probe: Tex6 seeds were supplied by Dr Don White at the University of Illinois at Urbana, which was an inbred selfed from southern corn cv. 'Whitemaster' (PI 401763). Tex6 was grown in a field screened movable greenhouse with irrigation as needed. Ears were self-pollinated and dated individually. The whole ears were collected at 5-day intervals from 25 to 45 DAP in the early morning. Three plant-ears were collected for each sample and there were three replicates for each at 25, 30, 35, 40 and 45 DAP. Ears were frozen in liquid nitrogen and stored at −80°C until used.

Total RNA was extracted from kernels using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with some modification as suggested in the production manual such as SAS (1%) used in the first step to separate starch from RNA (Prescott and Martin 1987) before TRIzol extraction to fit high starchy kernels, and three technique replications of single-ear were adopted at each sample. Three biological replications were implemented at each stage. Poly(A^+) RNA was isolated from total RNA using DynaBeads Oligo $(dT)_{25}$ (Dynal, Oslo, Norway) according to the manufacturer's instructions. Fluorescent-labelled cDNA probes were prepared using the indirect labelling method. Four micrograms of high quality poly(A^+) RNA was used to synthesize cDNAs in the presence of 5-(3-aminoallyl)-2′deoxyuridine-5′-triphosphate (AA-dUTP) (Amersham Biosciences, Piscataway, NJ, USA) using Superscript II reverse transcriptase (Invitrogen). The AA-cDNA probes of kernels were coupled with Cy3 monoreactive dye. The un-incorporated free dyes were removed using QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA).

Microarray hybridization and washing: Maize (*Zea mays* L.) oligo-nuleotide microarray, version 1.2 with approximately 58 000 70-mer oligo-nucleotide probes on a set of two slides, was purchased (http://www.maizearray.org) and used in these experiments. Hybridization and washing procedures were followed based on the recommended protocol. Briefly, the slides were re-hydrated over a water bath (50°C) for 5 s, and dried on a 45°C heating block for 5 s. Cy3-coupled cDNA probes with hybridization mixture were distributed equally for two slides, which the hybridization mixture consisted of 2x SSC, 0.08% (W/V) SDS, 6% (V/V) liquid block (Amersham Biosciences). The slides were placed in a hybridization chamber and transferred to the hybridization oven at 55°C for 12–14 h. The slides were washed in 2x SSC and 0.1% (w/v) SDS with gentle shaking, which was preheated to 55°C. After 5 min of washing, the slides were further washed in 0.5x SSC and 0.05x SSC for 5 min, respectively, at room temperature. The slides were spun dried and immediately scanned.

Microarray data analysis: Hybridized and washed slides were scanned using a ScanArray Express scanner (Perkin Elmer, Waltham, MA, USA) for Cy3 at 532 nm with a resolution of 10 μm per pixel, generating individual TIFF images. Signals of different slides were initially normalized during the image scanning process by adjusting the photomultiplier tube (PMT) based on positive controls on the slides. Local mean background was subtracted from the mean-based intensity for each spot. Two criteria were used for selecting positive spots, (Signal − Background) > 400 and (Signal/Background) > 2. Differentially expressed genes were identified by performing a one-way ANOVA (cutoff on $P < 0.05$) for gene identification with significant expression and a Benjamini and Hochberg (1995) multiple testing correlation was applied with a false discovery rate (FDR) of 0.05 such that 5% of the genes identified in a test are likely to be falsely identified. Separate ANOVAS were performed to identify genes that were differentially expressed among developmental stages. The acquired data were further analysed using GeneSpring (Silicon Genetics, Redwood City, CA, USA), *K*-means clustering ($k = 9$) for gene expression patterns, and condition tree for sample stage correlation based on gene expression (change correlation). The intensity of spots within a single slide was normalized by comparing with the internal reference gene GAPDH (MZ00023628), and the normalized gene was described as relative expression intensity (REI). Gene functional annotation was performed by using the Gene Ontology identifier (GO ID) assigned to this oligo by MOAP associated with the microarray slides, and by BLASTX with *E*-value < 10 as criterion to identify putative function of a gene.

Microarray data validation and qRT-PCR: Thirty genes with the expression patterns of up-regulation, down-regulation, or no-change in the microarray analysis were selected for quantitative analysis using one-step qRT-PCR. Total RNA from a single-ear was treated with DNase (Qiagen), and subsequently purified with RNeasy Cleanup Kit (Qiagen). To decrease replicated experimental variation at each sample stage, the three purified RNA ear-samples from were pooled equally for qRT-PCR. Three experimental technical replications were performed for each sample stage to assess the reproducibility, and the mean of the three replications was used to calculate relative expression quantitation. One-step qRT-PCR was performed using the QuantiTect SYBR green RT-PCR kit (Qiagen) according to the manufacturer's instructions. The total volume of reaction was 25 μl consisting of SYBR green RT-PCR master mix, QuantiTect RT mix, and 0.5 μM of each primer. Gene-specific primers were designed using OligoPerfect Designer (Invitrogen), and amplicons were designed to be < 150 bp. PCR assay was carried out with SYBR Green system in a DNA Engine Opticon (MJ Research, Waltham, MA, USA). Cycling parameters were set up according to the recommendation of QuantiTect SYBR green RT-PCR kit. Melting curves were run immediately after the last cycle to examine if the measurements were influenced by primer–dimer pairs.

The amplification curve was generated after analysing the raw data, and the cycle threshold (C_T) value was calculated based on the fluorescence threshold as 0.01. GAPDH was used as a 'house keeping' control gene in the original production of maize oligo-microarray. The expression of GAPDH across time was very constant using real-time PCR, and was used as an internal reference in this study, and the primers were 5′-ACTGTTCATGCCATCACTGC as forward primer and 5′-GAGGACAGGAAGCACTTTGC as reverse primer. The 'delta-delta C_T' ($2^{-\Delta\Delta C_T}$) mathematical model was used for description and comparison of the relative quantification of gene expressions between samples (Livak and Schmittgen 2001). Therefore, the amount of target gene in test sample was given by $R = 2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_{T\text{test sample}} - \Delta C_{T\text{reference sample}}$, $\Delta C_{T\text{sample}} = C(T)_{\text{test gene}} - C(T)_{\text{reference gene}}$. The final value of relative quantitation was described as fold change of gene expression in the tested sample compared with the reference sample.

Results

Overview of gene expression profiles in Tex6 kernels

We monitored gene expression profiles using maize oligo-array. The positive spots were selected based on the filter criteria, (Signal − Background) > 400 and (Signal/Background) > 2. There were total of 8497 genes with unique accession IDs detected in at least one sample stage. Of the total expressed genes, 4247 of them were common and present in all sample stages. The total positive genes, genes with known function, and unique genes in different stages were analysed for each sample stage (Fig. 1). The total number of expressed genes was decreasing when kernels were maturing. In general, the genes with known function counted for about 50% of total expressed genes at each stage, and the specific genes counted for about 7% at 25 DAP to < 2% at 45 DAP (Fig. 1).

Using the total positive genes detected as gene list, we monitored gene expression variations based on REI. The condition tree of hierarchical analysis reveals the relationship of different stages based on REI changes (Fig. 2). Three clusters were formed, 25 and 30 DAP, 35 DAP alone, and 40 and 45 DAP. The genes in same cluster had similar expression pattern. Expression characters at 35 DAP were closer to cluster of 25 and 30 DAP.

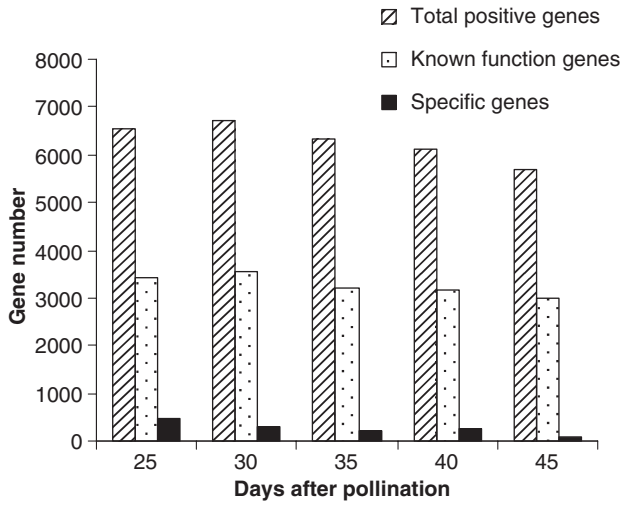

Fig. 1: The trends of expressed genes. Total numbers of expressed genes, genes with known function, and specific genes in different stages of developing kernels of Tex6 from 25 days after pollination (DAP) to 45 DAP

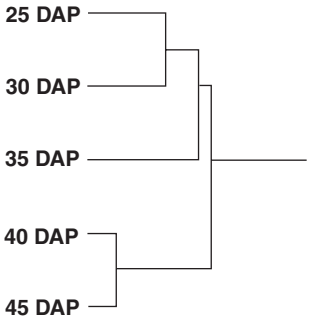

Fig. 2: Condition tree. Developmental stage correlation based on gene expression changes in maize developing kernels of Tex6. Similarity measure was based on expression change correlation, and gene expression value was $\log_2$ (normalized spot intensity). GAPDH (MZ00023628) was used as reference gene

The gene expression profile in kernels at 25 DAP is described here as an example. There were a total of 6349 genes with unique IDs detected with positive signals. However, the number we acquired did not represent the unique expressed genes. In the GAL (Gene Array Lists) file provided by MOAP, we found many genes with more than one ID, for example 19 IDs for 19 kDa α-zein B1. To identify gene function and corresponding functional classification, we used batch function (GO) search against MOAP and TIGR databases. For those genes matching the databases, GO terms were assigned based on the highest significant similarity score by using a cutoff value of $E < 10$. There were 3599 genes functionally annotated, and 1241 of them were unique genes which could match with one of GO slim catalogues with biological process, molecular function or cellular component. Examination of 654 genes, which functions belong to biological process (Fig. 3), revealed that a significant portion of the genes was involved in cellular and metabolic processes. Of the three metabolisms of carbohydrate, lipid and protein, which listed separately with other total metabolisms, protein metabolism showed larger proportion (Fig. 3).

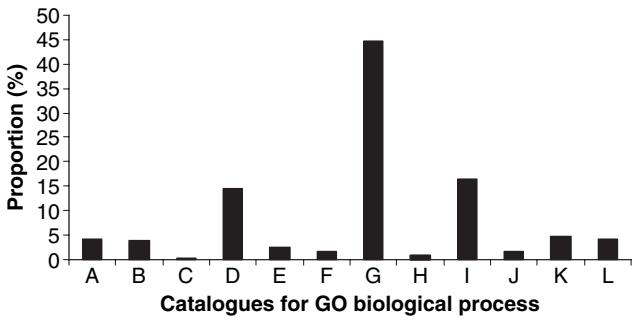

Fig. 3: Functional annotation and catalogues. There were a total of 654 genes with known biological process in maize developing kernels of Tex6 at 25 days after pollination. (a) Carbohydrate metabolism, (b) cell communication, (c) cell death, (d) cell growth or maintenance, (e) development, (f) lipid metabolism, (g) metabolism, (h) photosynthesis, (i) protein metabolism, (j) response to endogenous stimulus, (k) response to external stimulus, (l) response to stress

Difference of gene expression in Tex6 kernels

To explore the time-course changes of gene expression, gene expression patterns were analysed based on *K*-means clustering for comparison of values in $\log_2$ ratio. To calculate the ratio, the samples at 25 DAP were used as a reference, and samples from other stages were compared to the reference (Fig. 4). The total expression genes of 8497 in all stages were clustered into nine sets with different individual genes in each set, and the gene expression patterns were displayed as correlation plots (Fig. 4). If up-regulation, down-regulation, no-change, bell-shaped and U-shaped were used to describe the total patterns, the sets 1 and 4 would be classified as up-regulation and no-change profiles, the sets 2, 5 and 8 as down-regulation and no-change profiles, the sets 6 and 7 as U-shaped, and the sets 3 and 9 as bell-shaped pattern. From the continuous graphs of expression patterns, we could identify two interesting stages, 35 and 40 DAP, in which 40.3% of expression genes reached the lowest levels of expression at 35 DAP (sets 5, 6, 7) and the expression leaped up after 35 DAP (Fig. 4). About 82.5% of expression genes reached either the highest or lowest levels at 40 DAP (set 2, 3, 4 5, 6, 7, 9), and kept the status steadily after 40 DAP. From the gene expression patterns and the condition tree analysis (Figs 2 and 4), we could conclude that the 40–45 DAP was a special time-point which kernels entered a unique physiological phase.

Starch metabolism

As one of three main components, expression of several genes in starch biosynthesis pathway was tracked in these stages (Fig. 5). The results indicated that genes related with starch metabolism could be divided into two groups. Those tended to decrease in expression such as amylose extender starch-branching enzyme (MZ00007416), phosphoglucomutase (MZ00041470), starch synthase 2 (MZ00007657) and α-amylase inhibitor 5 (MZ00031962) (data not shown). After 40 DAP, the REI of these genes was very low (REI < 0.5). The second group of genes generally tended to increase and maintained a high expression intensity such as ADP glucose pyrophosphorylase (MZ00036672), fructokinase (MZ00 049604), putative amylase (MZ00016797) and putative sugar transporter (Fig. 5). The exception is Brittle-1 protein, which kept low expression without change during these stages.

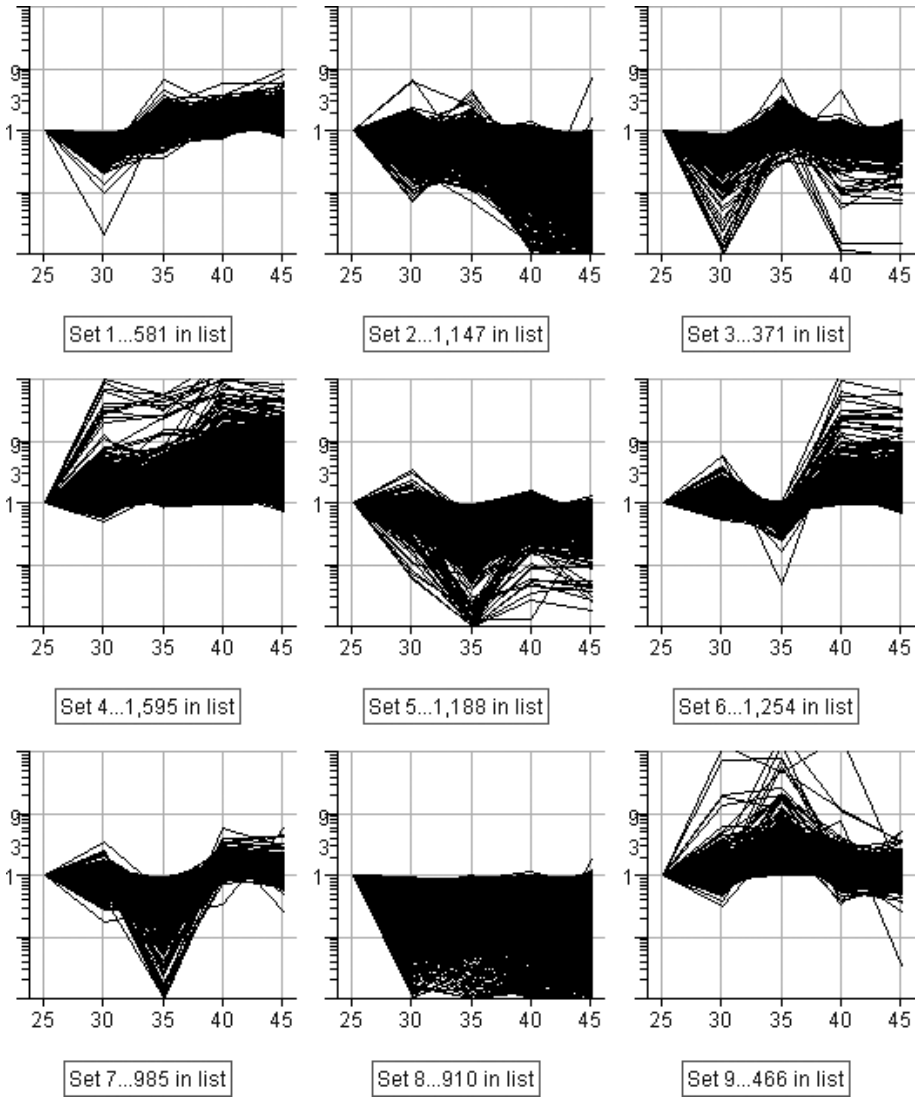

Fig. 4: Expression patterns. All positive genes in different stages of developing kernels of Tex6 based on *K*-means analysis. GAPDH (MZ00023628) was used as reference in each stage to normalize spot intensity. The expression data at 25 days after pollination (DAP) was used as reference base-line for comparison with other stages. *K*-means analysis: cluster set = 9, similarity measure = standard correlation. *Y*-axis = $\log_2$ ratio (normalized spot intensity in each sample stage/normalized spot intensity at 25 DAP). *X*-axis = DAP. Legend under each cluster shows gene number in the corresponding set

Lipid metabolism

The accumulation of storage lipid is located within the maize embryo. Several genes in fatty acid biosynthesis in maize kernel were expressed with high levels before 20 DAP (Lee et al. 2002). In this study, the REI of several genes in fatty acid and triacylglycerol biosynthesis was analysed. The results revealed that the REI of these genes was lower (average REI < 0.2), and indicated that lipid synthesis was down-regulated during these stages. But several other genes in lipid metabolism had higher expression levels, such as 1-acyl-glycerol 3-phophate acyltransferase (MZ00048303) with the highest REI (30.93), lipase (MZ00033464) REI (15.65), lipoxygenase (MZ00002269) REI (3.03) and phospholipids/glycerol acyltransferase-like protein (MZ00050640) REI (4.53). Among these genes related with lipid metabolism, those with oil body formation and storage, such as oleosin 17 (MZ00027573), and oleosin Zm-I (16 kDa) (MZ00024290), tended to increase in expression and oleosin Zm-II (18 kDa) (MZ00015939) stays no-change (Fig. 6).

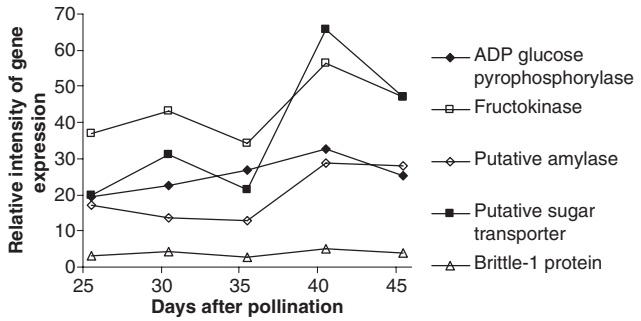

Fig. 5: Temporal patterns. Expression levels of some genes involved in starch biosynthesis/metabolism pathway in developing Tex6 kernels

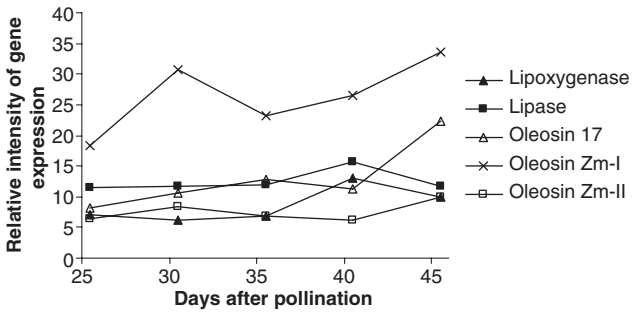

Fig. 6: Temporal patterns. Expression levels of some genes in lipid storage and metabolism pathway in Tex6 developing kernels

Storage protein genes

Although albumins, globulins, prolamins and glutelins are found in maize kernel, the predominate storage proteins in maize are globulin and prolamins which are known as zeins, including α-, β-, γ- and δ-zein families (Woo et al. 2001). On the array gene list, there are a total of 315 gene IDs annotated as storage proteins. To evaluate storage protein metabolism in Tex6 kernels, the 315 sequences were selected to compare their REIs. The gene family members with the highest signal intensity in each classification were listed in Table 1. The results indicated that all of the zein members had higher REIs before 40 DAP (Table 1). The REIs of zeins were decreased dramatically after 40 DAP. In comparison with zeins, however, the expression trends of globulin members were different (Table 1). α-Globulin tended to decrease and reached the lowest levels at 40 DAP. The expression of globulin-1 was increasing when kernels were growing and maturing, while globulin-2 maintained at a relatively high expression level (Table 1).

Defence-related genes

Through function analysis of expressed genes, we identified 823 defence-related genes, including disease resistance, stress response and reactive oxygen species (ROS) scavenger. Gene expressions were compared among all sampling stages (25, 30, 35, 40, 45 DAP). Because of the critical time for aflatoxin contamination, the comparison of these defence-related genes was performed between 45 and 25 DAP (Fig. 7A). If twofold change of ratio was used as criteria, there were about 84% defence-related genes expressed as patterns of up-regulation or no-change. One-way ANOVA was also performed to identify genes with significant expression (Fig. 7a). Of the 823 defence-related genes, the expressions of 211 genes were significantly different at 45 DAP ($P < 0.05$), and 134 of them were up-regulated. In the group of up-regulated genes, same genes with different IDs (fragments) or different family members were found to have different P-values or fold changes of expression. Only those with the lowest P-values were listed in Table 2.

Many defence-related genes expressed significantly higher at 45 DAP (Table 2). These genes have been reported as different pathogenesis-related (PR) gene families. The gene with the highest expression change at 45 DAP in comparison with 25 DAP was PR4 (MZ00043658) with 42 times, and more than 10 different genes expressed 10 times higher (Table 2). Phytoalexins have been known as important defence components in plants, and phenylalanine ammonia-lyase (PAL) is a main pathway to produce these substances. Some genes in PAL pathway were observed as up-regulation or no-change but maintaining at a high expression magnitude, such as chalcone synthase C2 (MZ00004400), cinnamoyl-CoA reductase (MZ00036801), cinnamyl alcohol dehydrogenase (MZ0000 4221), flavonoid glucosyl-transferase (MZ00051883) and phenylalanine ammonia-lyase (MZ00014291).

Among the stress-response genes (Table 2), desiccation tolerance genes are predominant, such as late embryogenesis abundant (LEA) proteins, lipoxygenase, low molecular weight heat shock proteins, dehydrin, lipid transfer protein, abscisic acid inducible gene, antifreeze proteins and ion channels. These genes expressed much higher at 45 DAP than those at 25 DAP. ROS are known as toxic products of normal metabolic functions as well as environmental stress in plants. ROS also are known as an important component in plants in response to biotic and abiotic stresses (Apel and Hirt 2004). The genes that can stimulate ROS production were identified in this study as up-regulation, such as respiratory burst oxidase homologs (MZ00036049) and peroxidase (MZ00050684). Antioxidants are responsible for maintaining ROS at a proper

Table 1: Expression levels of storage protein genes in maize developing kernels of Tex6

Gene ID	Gene name	Gene expression intensity[1]				
		25 DAP	30 DAP	35 DAP	40 DAP	45 DAP
MZ00041006	10 kDa zein precursor	48.57	42.55	23.12	3.58	2.44
MZ00037562	15 kDa β-zein	64.61	72.67	51.53	11.92	8.01
MZ00038146	16 kDa β-zein precursor	43.11	33.52	12.50	4.24	2.29
MZ00035180	18 kDa δ-zein	57.65	59.53	23.75	5.84	2.10
MZ00040886	19 kDa α-zein precursor	94.48	82.44	76.45	15.88	7.89
MZ00039295	19 kDa α-zein B1	79.87	78.37	35.76	7.59	5.06
MZ00039410	19 kDa α-zein B5	62.56	51.84	27.38	3.86	3.00
MZ00043007	19 kDa α-zein D1	52.51	54.64	13.16	3.22	2.09
MZ00041233	19 kDa α-zein D2	36.05	43.40	22.71	5.66	2.32
MZ00039403	Mutant 19 kDa S15P α-zein	77.79	83.67	57.71	17.04	10.37
MZ00035491	22 kDa α-zein 1-42	81.52	102.78	56.54	15.74	11.74
MZ00038250	22 kDa α-zein precursor	94.38	88.22	88.92	24.84	14.57
MZ00039759	22 kDa zein	81.23	79.81	46.60	14.14	10.53
MZ00043555	22 kDa α-zein 1	70.86	92.26	56.08	17.04	15.91
MZ00041020	22 kDa α-zein 4	99.30	70.16	43.00	17.27	10.57
MZ00042847	22 kDa α-zein 5	81.79	89.06	58.24	22.21	21.07
MZ00038256	27 kDa γ-zein	75.71	75.27	106.54	26.31	16.06
MZ00041168	50 kDa γ-zein	68.86	87.68	74.08	16.00	6.73
MZ00007618	Zein	85.71	75.82	43.78	7.68	2.99
MZ00038131	Zein floury2	85.23	88.79	63.01	15.74	10.18
MZ00040895	Zein zA1	82.87	80.21	41.09	15.57	5.88
MZ00040883	Zein zd1	98.88	79.02	67.75	20.82	9.60
MZ00016312	α-Globulin	8.65	9.53	4.25	1.55	1.27
MZ00040577	Globulin-1	2.04	3.32	3.19	5.38	10.51
MZ00016598	Globulin-2	8.30	10.50	7.18	6.04	7.51

ID, gene identification number; DAP, days after pollination.
[1]Mean of replicate experiments of selected gene normalized by reference gene GAPDH.

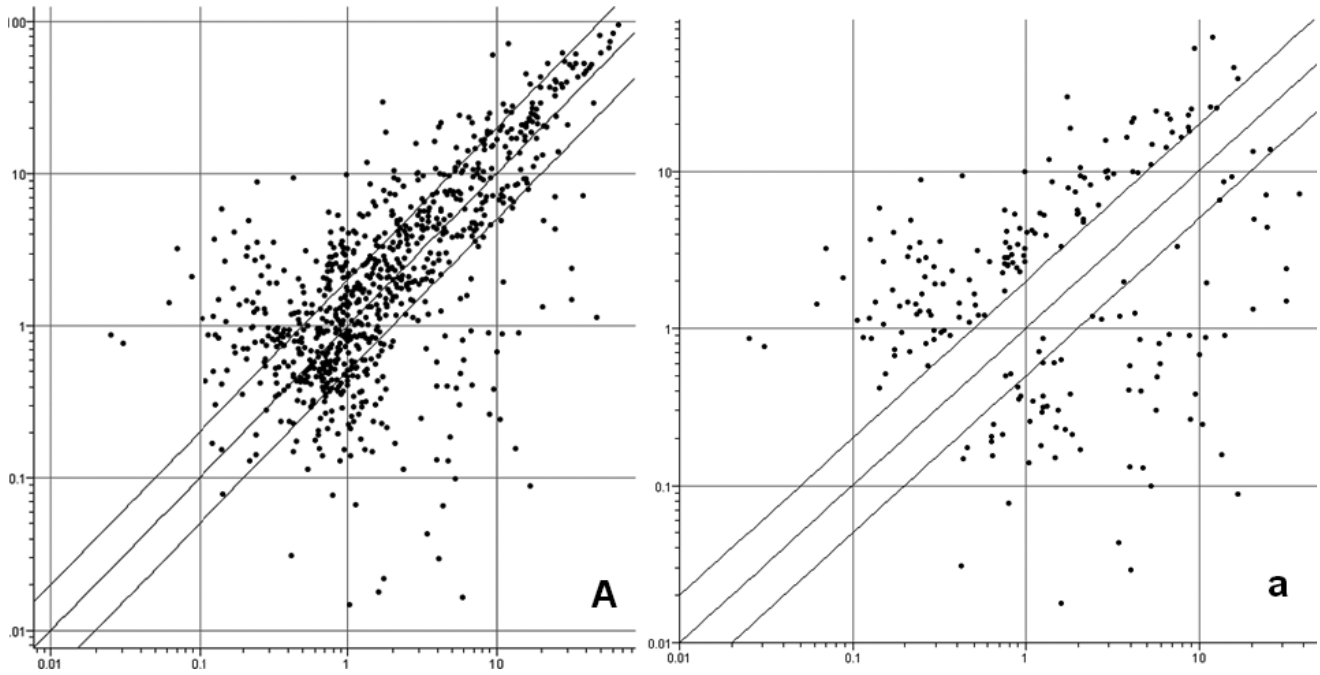

Fig. 7: Scatter plots. Total 823 expressed genes (A) and 211 significantly expressed defense-related genes (a) in maize developing kernels of Tex6 at 45 days after pollination (DAP) compared with 25 DAP. The significance of expression was based on one-way ANOVA analysis at $P < 0.05$. Spot value = $\log_2$ ratio, *X*-axis = normalized spot intensity at 25 DAP, *Y*-axis = normalized spot intensity at 45 DAP. Lines were set to show two fold changes of ratio

level and preventing oxidative damage. Scavengers of antioxidant genes were detected at 45 DAP, such as ascorbate peroxidase, glutathione S-transferases and superoxide dismutase (Cu–Zn).

Real-time qRT-PCR to validate microarray

Thirty genes with up-regulated, unchanged or down-regulated patterns in microarray study were selected for specific primer design for qRT-PCR. Relative quantitative method delta-delta C_T ($2^{-\Delta\Delta C}{}_T$) was used to describe expression patterns of selected genes by comparing the gene expression levels at 45 DAP with 25 DAP. The relative quantitation comparisons based on C_T values from the test samples (45 DAP) and the control samples (25 DAP) were calculated as the algorithm $R = 2^{-\Delta\Delta C}{}_T$. Generally, *R*-value > 2.00 was described as up-regulation, *R*-value < 0.50 as down-regulation, and $2.00 \geq R\text{-value} \geq 0.50$ as no-change. The results indicated that the expression levels measured by qRT-PCR of the majority genes, up-regulated (+ +) and unchanged (+ −), reproduced the microarray study very well (Table 3). The exceptions were those of down-regulation (− −), such as ABA-induced protein (MZ00012173) and lipid transfer protein (MZ00013624).

Discussion

In this study, we conducted microarray analysis of temporal profiles of gene expression in Tex6 kernels. The total numbers of expressed genes were decreasing when kernels were growing to maturity. Genes in several pathways were monitored. The expression of defence-related genes was significant. Over 83% of these genes were expressed as up-regulated or unchanged with high expression levels even after 40 DAP. Thirty genes from microarray study were validated by qRT-PCR.

Maize kernel development is an important biological process. Typical maize kernels contain about 4% oil, 9% protein, 73% starch and 14% other constituents such as fibre (Lambert et al. 2004). The deposition of reserves in maize kernel usually happens at grain filling phase (Herman and Larkins 1999). In maize public EST database, the majority of seed ESTs came from 25 DAP and earlier. Lee et al. (2002) reported that the changes of individual mRNA accumulation during maize kernel development after 25 DAP were much smaller, which agrees with our data that the total numbers of genes were decreasing as the kernels were growing to maturity. However, our investigation of gene transcript profiles has shown that metabolic activities were still active after 25 DAP. The genes had different expression patterns associated with different metabolism pathways.

Our interest was to study the gene expression patterns in Tex6 and identify some unique genes that could be used in germplasm assessment. From the results of selected genes in starch, lipid and protein metabolisms, the expression trends were similar to the results of others (Shannon et al. 1998, Lee et al. 2002, Lambert et al. 2004). The qRT-PCR results of selected genes have validated microarray data and there were exceptions which could be explained by several aspects which result in the bias. Cross-hybridization between family members or homologous sequences might be a possible reason resulting in the bias.

Our initial thought reasoned that the expression of some defence-related genes might be involved in the tolerant traits in Tex6. There were more than 800 defence genes detected in kernels of Tex6, and some of them expressed significantly higher after 40 DAP (Table 2). Previous studies reported that maize kernels have different classes of defence/stress-related proteins with known antifungal activity and these proteins might contribute to a general or specific resistance trait (Guo

Table 2: Up-regulated defence-related genes in three categories in maize developing kernels of Tex6 at 45 days after pollination (DAP) compared with 25 DAP

Gene ID	P-value[1]	FC[2]	Putative function[3]
Disease resistance			
MZ00029691	2.80E-03	10.18	Allyl alcohol dehydrogenase (*Nicotiana tabacum*)
MZ00031167	6.06E-04	25.2	Antifungal zeamatin-like protein (*Oryza sativa*)
MZ00030174	4.86E-03	5.52	β 1,3-glucanase (*Oryza sativa*)
MZ00040645	1.29E-02	2.12	1,3-β-glucanase PRm 6b (Zea mays)
MZ00041326	7.78E-04	36.55	Bowman-Birk type trypsin inhibitor (*Triticum aestivum*)
MZ00043035	3.25E-03	7.66	Chitinase (*Zea mays*)
MZ00041278	4.46E-05	6.51	Chitinase A (*Zea mays*)
MZ00041277	2.90E-03	10.51	Chitinase-B1 (*Sorghum bicolor*)
MZ00004170	7.73E-03	2.90	Chitinase III (*Oryza sativa*)
MZ00036158	3.24E-02	3.91	Chitinase IV (*Arabidopsis thaliana*)
MZ00036801	3.15E-03	6.82	Cinnamoyl-CoA reductase (*Oryza sativa*)
MZ00050685	3.73E-02	3.24	Cysteine proteinase (*Hordeum vulgare*)
MZ00024534	2.77E-02	2.96	Cysteine proteinase component of protease-inhibitor complex (*Zea mays*)
MZ00039782	2.77E-02	2.53	Cysteine proteinase inhibitor precursor (*Zea mays*)
MZ00043560	9.53E-04	4.32	Endochitinase B precursor (*Zea mays*)
MZ00031909	2.88E-03	10.79	Ethylene-forming-enzyme-like dioxygenase-like protein (*Oryza sativa*)
MZ00039425	1.36E-02	2.93	γ-Zeathionin 1 (*Zea mays*)
MZ00009851	4.68E-02	2.73	γ-Zeathionin 2 (*Zea mays*)
MZ00031156	3.25E-03	8.43	GA 2-oxidase (*Oryza sativa*)
MZ00000286	3.50E-04	23.22	Glucan endo-1,3-β-glucosidase GII precursor (*Hordeum vulgare*)
MZ00040560	2.18E-04	7.61	Glucose starvation-induced protein precursor (*Zea mays*)
MZ00044002	6.80E-4	6.26	Licheninase precursor (*Triticum aestivum*)
MZ00014150	4.84E-02	3.77	3-Ketoacyl-CoA thiolase-like protein (*Oryza sativa*)
MZ00016209	6.06E-03	5.20	Metallothionein II (*Zea mays*)
MZ00037932	2.88E-02	2.24	Metallothionein-like protein (*Quercus suber*)
MZ00013363	7.68E-03	6.33	Metallothionein-like protein 1 (*Zea mays*)
MZ00042895	7.57E-03	3.47	Metallothioneine type 2 (*Hordeum vulgare*)
MZ00009229	9.49E-03	2.78	NBS-LRR-like resistance protein 5 (*Avena strigosa*)
MZ00036381	3.05E-02	4.34	33 kDa oxygen evolvingprotein of photosystem II (*Oryza sativa*)
MZ00042168	1.71E-03	11.49	Pathogenesis-related protein 1 (*Zea mays*)
MZ00043658	1.10E-03	41.56	Pathogenesis-related protein 4 (*Hordeum vulgare*)
MZ00013547	1.77E-02	24.20	Pathogenesis-related protein 5 (*Zea mays*)
MZ00033697	8.66E-03	7.48	Pathogen-related protein 10 (*Oryza sativa*)
MZ00018153	5.52E-04	34.89	Pathogenesis-related protein PRMS precursor (*Zea mays*)
MZ00016495	4.87E-05	29.49	Permatin precursor (*Avena sativa*)
MZ00014291	2.47E-02	2.80	Phenylalanine ammonia-lyase (*Zea mays*)
MZ00050684	3.35E-02	3.52	Peroxidase (*Oryza sativa*)
MZ00036049	1.11E-02	3.21	Respiratory burst oxidase homolog (*Solanum tuberosum*)
MZ00008983	4.54E-03	10.23	Ribonuclease III (*Oryza sativa*)
MZ00041005	1.66E-02	2.22	Subtilisin/chymotrypsin inhibitor (*Zea mays*)
MZ00013291	4.87E-03	4.53	Subtilisin-like protease (*Oryza sativa*)
MZ00036006	1.97E-02	4.15	Subtilisin-like serine protease (*Oryza sativa*)
MZ00049943	1.17E-02	3.14	Serine protease (*Oryza sativa*)
MZ00041513	1.76E-02	3.62	Wound-induced protease inhibitor (*Zea mays*)
MZ00014433	4.82E-04	6.55	Xylanase inhibitor protein (*Triticum turgidum*)
MZ00017927	9.40E-04	4.09	Zeamatin precursor (*Zea mays*)
MZ00018112	1.11E-02	3.89	Zeta-carotene desaturase (*Zea mays*)
Stress response			
MZ00033933	3.46E-02	4.76	Antifreeze glycoprotein precursor (*Oryza sativa*)
MZ00037908	4.72E-03	2.20	Abscisic acid inducible gene (*Zea mays*)
MZ00048447	3.41E-02	2.33	Auxin induced protein (*Oryza sativa*)
MZ00038054	1.15E-02	2.69	Auxin response factor 1 (*Oryza sativa*)
MZ00043478	1.02E-02	4.37	Auxin response factor 2 (*Oryza sativa*)
MZ00013901	4.30E-04	9.41	Auxin-repressed protein (*Robinia pseudoacacia*)
MZ00035042	4.33E-03	17.99	Class I heat shock protein (*Glycine max*)
MZ00038909	4.47E-02	2.10	Cold acclimation protein WCOR825 (*Triticum aestivum*)
MZ00040421	2.86E-02	3.04	Cold-regulated protein (*Hordeum vulgare*)
MZ00014527	1.28E-02	3.26	Cytosolic heat shock protein 90 (*Hordeum vulgare*)
MZ00007449	1.15E-03	8.22	Dehydrin (*Hordeum vulgare*)
MZ00046901	6.80E-03	14.70	Dehydrin 6 (*Hordeum vulgare*)
MZ00025109	1.74E-03	17.44	Dehydrin DHN1 (M3) (RAB-17 protein) (*Zea mays*)
MZ00006651	4.23E-02	2.56	D-type cyclin (*Zea mays*)
MZ00026333	2.03E-03	3.47	Embryonic abundant protein EMB5 (*Zea mays*)
MZ00044526	6.79E-03	10.80	Embryo-specific protein (*Oryza sativa*)
MZ00035463	2.74E-02	2.90	Heat shock protein 17.2 (*Zea mays*)
MZ00015499	3.82E-02	2.36	Heat shock protein 22 (*Zea mays*)
MZ00038605	2.54E-03	4.40	Heat shock protein 82 (*Oryza sativa*)
MZ00050216	8.80E-03	2.04	Heat-shock protein (*Oryza sativa*)

Table 2: Continued

Gene ID	P-value[1]	FC[2]	Putative function[3]
MZ00017449	4.77E-03	5.37	Heat-shock protein class I, 18.6K (*Helianthus annuus*)
MZ00043232	8.88E-03	2.33	Hydroxyproline-rich glycoprotein DZ-HRGP (*Volvox carteri*)
MZ00028039	1.75E-04	6.03	Late embryogenesis abundant protein EMB564 (*Zea mays*)
MZ00009861	1.20E-02	11.32	Late embryogenesis abundant protein LEA14-A (*Oryza sativa*)
MZ00040778	5.59E-03	14.01	Late embryogenesis abundant protein, group 3 (*Zea mays*)
MZ00044877	6.83E-03	3.34	Lipoxygenase (*Zea mays*)
MZ00041610	1.34E-03	2.22	Non-specific lipid-transfer protein precursor (*Zea mays*)
MZ00017607	9.38E-03	2.80	NPH3 family protein (*Oryza sativa*)
MZ00031491	1.58E-02	5.37	70 kDa peptidylprolyl isomerase (*Triticum aestivum*)
MZ00022783	5.15E-03	2.94	Potasium transporter (*Oryza sativa*)
MZ00047122	5.85E-03	3.64	Potassium channel protein ZMK2 (*Zea mays*)
MZ00032472	2.97E-03	10.52	Proline-rich protein-like (*Oryza sativa*)
MZ00033881	1.92E-03	3.13	Rab28 protein (*Zea mays*)
MZ00000037	1.39E-02	5.44	Response regulator (*Zea mays*)
MZ00028489	1.26E-02	2.94	S276 (*Triticum aestivum*)
MZ00031538	8.81E-03	7.48	Salt tolerance protein (*Oryza sativa*)
MZ00041181	1.22E-02	5.90	Salt-stress root protein RS1 (*Oryza sativa*)
MZ00029810	1.44E-02	2.78	Stress inducible protein (*Oryza sativa*)
MZ00026357	1.32E-03	5.14	Stress-inducible membrane pore protein (*Bromus inermis*)
MZ00024937	1.39E-02	7.16	Wound inductive gene (*Oryza sativa*)
ROS scavenger			
MZ00041550	9.44E-04	2.22	L-ascorbate peroxidase (*Zea mays*)
MZ00015071	1.59E-02	7.18	Glutathione S-transferase (*Oryza sativa*)
MZ00054882	2.13E-02	2.46	Glutathione S-transferase GST7 protein (*Zea mays*)
MZ00024194	2.05E-02	2.24	Superoxide dismutase (Cu-Zn) 4 (*Zea mays*)

ID, gene identification number.
[1]One way-ANOVA analysis by comparing 45 DAP with 25 DAP.
[2]FC: fold changes as means of $\log_2$ ratio (expression intensity at 45 DAP vs. 25 DAP).
[3]Putative gene function, gene name with highly matched species from sequence homologous analysis provided by the MOAP (maize oligonuleotide array project).

Table 3: Validation of expression patterns of selected genes from microarray analysis using real-time qRT-PCR

Gene ID	Putative gene function	Microarray analysis[1]	Real time qRT-PCR[2]
MZ00037908	Abscisic acid inducible gene	+ +	10.91
MZ00013897	Chitinase	+ +	28.99
MZ00013823	Cysteine protease	+ +	7.42
MZ00040965	γ-Zeathionin 2	+ +	12.19
MZ00042394	Globulin-1S	+ +	14.61
MZ00016209	Metallothionein II	+ +	5.36
MZ00042357	Late embryogenesis abundant protein, group 3	+ +	23.48
MZ00041610	Non-specific lipid-transfer protein precursor	+ +	19.49
MZ00014291	Phenylalanine ammonia-lyase	+ +	11.50
MZ00039408	Proline rich protein	+ +	23.08
MZ00046334	Bax inhibitor-1	+ −	1.35
MZ00043559	Chitinase	+ −	0.90
MZ00000070	Drought inducible 22 kDa protein	+ −	0.94
MZ00048100	Early-responsive to dehydration stress protein	+ −	1.22
MZ00051831	Endo-1,4-β-glucanase	+ −	1.48
MZ00035860	GRAB1 protein	+ −	1.27
MZ00037250	Late embryogenesis abundant protein	+ −	1.48
MZ00013624	Lipid transfer protein	+ −	1.08
MZ00051962	Hydroxyproline-rich glycoprotein DZ-HRGP	+ −	0.78
MZ00049070	Small heat shock-like protein	+ −	0.71
MZ00012173	ABA-induced protein	− −	0.69
MZ00016313	α-Globulin	− −	0.43
MZ00024038	Hageman factor inhibitor	− −	0.31
MZ00040001	Lipid transfer protein	− −	0.74
MZ00039306	Metallothionein-like protein	− −	0.35
MZ00014523	NAM-related protein 1	− −	0.56
MZ00048862	Proline-rich protein	− −	0.57
MZ00025588	Proteinase inhibitor	− −	0.17
MZ00042045	Response regulator 6	− −	0.50
MZ00048061	Stress-related protein	− −	0.38

ID, gene identification number.
[1]Microarray analysis, results of the expression patterns of selected genes at 45 days after pollination (DAP) compared with 25 DAP after one-way ANOVA analysis ($P < 0.05$), +/− used to show gene expression trends in microarray analysis, + +, up-regulated, + −, unchanged ($P \geq 0.05$), − −, down-regulated.
[2]Real time qRT-PCR, results of relative quantitative qRT-PCR [$R = 2^{-\Delta\Delta C(T)}$] of selected genes at 45 DAP vs. 25 DAP. *R*-value > 2.00 as up-regulated, *R*-value < 0.50 as down-regulated, 2.00 ≥ *R*-value ≥ 0.50 as unchanged.

et al. 1997, Chen et al. 2001, 2002, 2004, Moore et al. 2004). *Aspergillus flavus* infection of maize kernels occurs earlier than aflatoxin accumulation in developing kernels (Payne 1998). Aflatoxin contamination occurs later in the maturation phase. Recent studies have demonstrated higher concentration of defence- or stress-related proteins in mature maize kernels of resistant genotypes compared with susceptible genotypes (Chen et al. 2002, 2004). Furthermore, in this study, we reported many defence-related genes detected. Also we observed several genes in some signal pathways, which were up-regulated along with some defence genes. For the purposes of selection of genes in the later stages of kernel development, we conducted this study to identify some unique genes that were up- or down-regulated in Tex6, which could be selected and used to produce a macroarray tool for germplasm assessment and evaluation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1: Total 8497 expressed genes in Tex6 developing kernels.
Table S2: Total 823 expressed genes in Fig. 7A.
Table S3: Total 211 defence-related genes in Fig. 7a.
Table S4: Primers used for validation of expression patterns of selected genes in Table 3 from microarray analysis using real-time qRT-PCR.
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